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Evolution of Developmental Control Mechanisms

An anterior medial cell population with an apical-organ-like transcriptional profile that pioneers the central nervous system in the centipede *Strigamia maritima*

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ABSTRACT

The apical plate of primary marine larvae is characterized by a common set of transcription factors comprising *six3*, *rx*, *hbn*, *nk2.1* and *FoxQ2*. It harbours the apical organ, a neural and ciliary structure with neurosecretory properties. Recent studies in lophotrochozoans have found that apical organ cells form the anterior tip of the developing central nervous system.

We identify an anterior medial tissue in the embryonic centipede head that shares the transcriptional profile of the apical plate of marine larvae, including nested domains of *FoxQ2* and *six3* expression. This domain gives rise to an anterior medial population of neural precursors distinct from those arising within the segmental neuroectoderm. These medial cells do not express *achaete scute homologue* in proneural clusters, but express *collier*, a marker for post mitotic cells committed to a neural fate, while they are still situated in the surface ectodermal layer. They then sink under the surface to form a compact cell cluster. Once internalized these cells extend axons that pioneer the primary axonal scaffold of the central nervous system. The same cells express *phc2*, a neural specific prohormone convertase, which suggests that they form an early active neurosecretory centre. Some also express markers of hypothalamic neurons, including *otp*, *vtn and vax1.*

These medial neurosecretory cells of the centipede are distinct from those of the *pars intercerebralis*, the anterior neurosecretory part of the insect brain. The *pars intercerebralis* derives from *vsx* positive placodal-like invagination sites. In the centipede, *vsx* expressing invaginating ectoderm is situated bilaterally adjacent to the medial pioneer cell population. Hence the *pars intercerebralis* is present in both insect and centipede brains, whereas no prominent anterior medial cluster of pioneer neurons is present in insects. These observations suggest that the arthropod brain retained ancestrally an anterior medial population of neurosecretory cells homologous to those of the apical plate in other invertebrate phyla, but that this cell population has been lost or greatly reduced in insects.

Introduction

There is a long history of debate as to whether the arthropod head retains structures homologous to the anterior, unsegmented tissue of annelids and other invertebrates. Morphological studies have in recent years tended to reject this idea, suggesting that the entire arthropod head is segmentally derived (Budd, 2002; Haas et al., 2001) (Note that we use the term arthropod to include hexapods, myriapods, crustaceans and chelicerates, but exclude onychophorans). New phylogenies have made any close correspondence between arthropod and annelid head organisation seem less likely (Aguinaldo et al., 1997; Dunn et al., 2008). Against this however, the conservation of transcription factor expression in the anterior regions of the most diverse animals has recently lead to the proposal that aspects of anterior patterning are conserved across, and even beyond, the bilateria (Lowe et al., 2003; Posnien et al., 2011; Sinigaglia et al., 2013; Steinmetz et al., 2010).

The morphology of the head in adult arthropods, as in other animals, shows complex adaptions to behaviour and life style. If we are to find remnants of any ancestral organisation that underlies this diversity, and is shared between widely disparate groups, it seems likely that this will be most evident during early embryogenesis, and reflected in the molecular specification of the first distinct territories and cell types to arise during head patterning. This approach has already led to a better understanding of evolutionary

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conserved regions in axial patterning (Lowe et al., 2003; Schilling and Knight, 2001; Steinmetz et al., 2010), and to the identification of evolutionarily related cell types in distant animal taxa (Arendt, 2008; Tessmar-Raible et al., 2007; Tomer et al., 2010). We have taken this approach to study the organisation of the head in a centipede, as representative of an ancient but hitherto poorly studied lineage of the arthropods.

Debate about the nature of the anterior body region of arthropods has a long history, focussing on the number of segments in the head (*e.g.* Rogers and Kaufmann, 1996), the homology of the different head segments between arthropod lineages (Damen et al., 1998; Haas et al., 2001; Scholtz and Edgecombe, 2006; Telford and Thomas, 1998) and the nature of the arthropod brain (Lichtneckert and Reichert, 2005; Urbach and Technau, 2003). Molecular markers for segment patterning, and in particular, the analysis of Hox gene expression domains (Damen et al., 1998; Hughes and Kaufman, 2002; Telford and Thomas, 1998), have largely resolved controversies about segment homologies in the post antennal region, but the structure of the most anterior part of the head and brain remains controversial.

The arthropod brain is classically divided into three units: the tritocerebrum most posteriorly, deriving from the intercalary segment in insects and myriapods, and from the homologous 2nd antennal segment of crustaceans; the deutocerebrum, deriving from the antennal segment of insects (1st antennal of crustaceans), and the protocerebrum, positioned most anteriorly. The protocerebrum comprises the ocular lobes, the mushroom bodies and the central complex, which includes the *pars intercerebralis* (Scholtz and Edgecombe, 2006; Strausfeld, 2012). The embryonic origin of the protocerebrum is from the pre-antennal head, but it has not been clear whether the embryonic pre-antennal region is one large territory of segmental origin (the ocular region) or might additionally comprise an anterior medial tissue. The presence of an anterior medial tissue, giving rise to parts of the central complex and the labrum, has been proposed on the basis of recent molecular work in the beetle *Tribolium castaneum* (Kittelmann et al., 2013; Posnien et al., 2011, 2009).

Recent support for the idea that the most anterior part of the head in arthropods and annelids may be homologous comes from studies of a homeobox transcription factor, *six3*, which is widely conserved across the animals. *Six3* is expressed in the apical plate of the annelid trochophore and the anterior medial head of several arthropods (Steinmetz et al., 2010), as well as in the anterior ectoderm of other bilaterian animals, and even in the larva of the cnidarian *Nematostella vectensis*. This suggests that a *six3* expressing anterior territory may have been inherited from the bilaterian ancestor (Sinigaglia et al., 2013; Steinmetz et al., 2011). If so, parts of the central nervous system that derive from the anterior medial head, and express *six3,* are likely to have a deep evolutionary origin.

Further studies on gene expression in free-swimming larvae of marine organisms have elucidated a conserved set of transcription factors characterising this most anterior (apical) region, comprising *six3*, *FoxQ2*, *nk2.1*, *rx* and *hbn* (Santagata et al., 2012; Sinigaglia et al., 2013; Steinmetz et al., 2010; Takacs et al., 2004; Tessmar-Raible et al., 2007; Wei et al., 2009; Yaguchi et al., 2008). Orthologues of *six3*, *rx* and *nk2.1* have also been shown to be involved in development of the vertebrate forebrain and hypothalamus (Lagutin et al., 2003; Lu et al., 2013; Muranishi et al., 2012; Ohuchi et al., 1999; Oliver et al., 1995; Tessmar-Raible et al., 2007), whereas *hbn* is missing from the vertebrate genomes (Mazza et al., 2010) and *FoxQ2* is not present in mammals (Shimeld et al., 2010) and has so far not been characterised in any vertebrate.

This apical territory of marine larvae harbours the apical organ, which is positioned centrally within nested domains of *FoxQ2* and *six3* (*e.g.* Santagata et al., 2012; Sinigaglia et al., 2013). Apical organs are larval sensory structures that include neurosecretory cells (Conzelmann et al., 2011; 2013). A recent study argues for homology of larval apical organs among animals that develop free-swimming marine larvae, including cnidarians (Marlow et al. 2014). In species that undergo a dramatic change of body plan during metamorphosis the apical organ is completely lost at the transition to the adult form (see for example Nielsen, 2005). By contrast, the polychaete annelid *Platynereis dumerilii* undergoes a gradual mode of metamorphosis and cells of the apical organ are partially maintained into late larval and adult stages. They produce pioneer neurons and are thought to form a nucleation centre for the developing nervous system of the animal (Marlow et al. 2014, Fischer et al. 2010).

Until now it has not been clear what parts of the arthropod brain derive from an anterior medial territory. One candidate is the *pars intercerebralis*, which has been shown to derive from the *six3+* territory in the insects *Drosophila* and *Tribolium*. The *pars intercerebralis* constitutes the anterior neurosecretory part of the central complex in insects (Boyan and Reichert, 2011; De Velasco et al., 2007; Posnien et al., 2011) and so might plausibly be homologous to the anterior neurosecretory brain centres in the lophotrochozoans and the hypothalamus of vertebrates (Hartenstein, 2006; Tessmar-Raible, 2007).

Both the annelid apical organ and the insect *pars intercerebralis* are located within the *six3+* territory at the anterior end of the the axonal scaffold. but the pars intercerebralis develops from bilateral ectodermal placodes at the lateral edges of the *six3+* territory (De Velasco et al., 2007; Steinmetz et al., 2010). The central anterior medial head of insects mainly gives rise to the labrum, a non-neural and probably appendicular structure (Posnien et al., 2011, 2009) (though others have interpreted the labrum as appendicular but of segmental origin [*e.g.* Boyan et al. 2002]). So far it is not clear whether the *pars intercerebralis* bears any further developmental or transcriptional similarity with the anterior neurosecretory cells that are part of the apical organ and connect to the anterior axonal scaffold of the marine larvae (Conzelmann et al., 2013; Fischer et al., 2010; Santagata et al., 2012; Tessmar-Raible et al., 2007).

Studies of the anterior medial head in insects are complicated by the fact that the insect head undergoes major structural rearrangements during development. This process is carried to an extreme in *Drosophila* and other *Diptera,* where the whole anterior head undergoes the process of head involution (Turner and Mahowald, 1979). In *Tribolium*, which is currently the major model for the genetic control of head development in insects (Kittelmann et al., 2013; Posnien et al., 2011, 2010; Schinko et al., 2008), the anterior medial region is a small region of tissue which comes to lie between the ocular lobes in early development, and forms the labrum anlagen, the anterior-most portion of the medial head (Kittelmann et al., 2013; Posnien et al., 2011).

Little work has been done on head regionalization and molecular specification of anterior brain structures in non-insect arthropods. Here we study these processes in a myriapod, the centipede *Strigamia maritima*. Myriapods are now recognised as an early branch of the mandibulate arthropods, which emerged basal to the pancrustacean (*i.e.* crustacean and insect) radiation (Regier et al., 2010; Rota-Stabelli et al., 2011) (but see (Friedrich and Tautz, 1995; Mayer and Whitington, 2009; Pisani et al., 2004) for alternative views). *Strigamia* is the first myriapod for which a sequenced genome is available (⟨http://www.ncbi.nlm.nih.gov/assembly/322118/⟩). The gene content of *Strigamia* is conservative; the genome contains a number of factors that have been lost from insect genomes. We could for example identify a clear homologue of *vax1* (Chipman et al., in press), a gene involved in development of anterior neurosecretory organs in vertebrates (Bertuzzi and Hindges, 1999; Bharti et al., 2011; Wataya et al., 2008), which is not present in insects (Tessmar-Raible, 2007).

The head field of *Strigamia* condenses during early development on the egg surface (Brena and Akam, 2012), allowing gene

expression to be visualised readily throughout the process of head patterning. This, together with the genome resources, makes *Strigamia* a good model for studying arthropod head development.

We show here that gene expression in the anterior medial region of the *Strigamia* head shares striking similarities with that in the apical territories of *Platynereis* and other marine invertebrate larvae, as well as with the forebrain/hypothalamic region of vertebrates. An early specified anterior-medial neurosecretory cell population shares a transcription factor profile with the apical cells of lophotrochozoan larvae, but is distinct from the *pars intercerebralis* known from insects. This cell group not only forms an active neurosecretory centre but also pioneers the axonal tracts of the centipede nervous system. We discuss the implications of these observations for the origin of neurosecretory brain centres, the developmental structure of the arthropod protocerebrum and the evolution of the arthropod nervous system.

Results

The anterior medial head of Strigamia maritima is a developmentally distinct territory that is demarcated by a set of conserved transcription factors

In *Strigamia*, the future head becomes visible shortly after the uniform blastoderm stage, as a field of cells that condenses towards the ventral anterior part of the forming embryo (Brena and Akam, 2012). As condensation progresses, this head field becomes sharply demarcated from the surrounding single layered epithelium of the dorsal field, a presumptive extra-embryonic territory (Fig. 1).

We have used molecular markers to define distinct regions within this condensing head. Many genes are expressed segmentally in the more posterior part of the head field, defining the pre-antennal, antennal, intercalary and gnathal segments (*e.g. buttonhead*/*SP5*, figure 1AB; *sloppy paired*, *Pax6* (Supplementary Fig. S4)). (Note that *Strigamia*, like all geophilomorph centipedes, lacks eyes. We therefore refer to a pre-antennal segment rather than an ocular segment, which is the term used to describe the corresponding region in other arthropods. Despite the lack of eyes, this region in Strigamia is innervated by a prominent neuropil (Fig. 2F)).

The most anterior part of the head does not express these characteristic markers of segmented tissue (Fig. 1A and B) but does express the conserved anterior patterning genes *six3* (see Steinmetz et al., 2010, and Fig. 1J and K), *FoxQ2*, *nk2.1* and *hbn* (Fig. 1C–F). We refer to the tissue defined by the expression of these genes as the anterior medial region (AMR). *Six3* is expressed throughout the anterior of the early head field, in a domain directly anterior to pre-antennal *otx* expression, (Fig. 1J). *FoxQ2* marks the central part of the condensing AMR (Fig. 1C). *nk2.1* is also expressed centrally in the AMR but more posteriorly than *FoxQ2*. The anterior part of the ring-like *nk2.1* domain overlaps with *FoxQ2* expression (Fig. 1D, C, L). The homeobox gene *hbn* is expressed in an arch covering the anterior rim of the AMR and extending into the dorsal field (Fig. 1E). The posterior limit of *hbn* is directly adjacent to or slightly overlapping with the anterior limit of *nk2.1* expression. The factor *rx*, part of the conserved *rx-hbn-otp* gene cluster (see Supplementary Fig. S3B) is not expressed at the very early developmental stages, but slightly later, at early segmentation stages, it is expressed in a pattern similar to that of *hbn* within the anterior head (Fig. 1G).

The AMR becomes positioned between the two halves of the pre-antennal region by a morphogenetic re-arrangement of the anterior head

During the early stages of head condensation the AMR is located anterior to the pre-antennal region; only the *nk2.1* positive part of the AMR reaches between the two halves of the pre-antennal region (Fig. 1A, D). The mouth opening/stomodaeum develops in the centre of this *nk2.1* positive domain. With ongoing condensation and development of the head field, the AMR converges medio-laterally and becomes enclosed laterally by the pre-antennal lobes (Fig. 1B and M). During this process the expression domains of the anterior medial markers become more condensed (Fig. 1F and I). In the head of mid-segmentation and later stage embryos the pre-antennal domains reach to the anterior tip of the head, but are situated lateral to the AMR (see Fig. 1N). However, based on the embryonic origin of the AMR from a more anterior position, this tissue can clearly be identified as the most anterior part of the head.

In summary, we find an anterior medial tissue in the head of *Strigamia* where the factors *six3*, *FoxQ2*, *nk2.1*, *rx* and *hbn* are all expressed in partially overlapping domains (Fig. 1C and L). All of these factors have been shown to be expressed in the apical territory of the polychaete annelid *Platynereis dumerilii* (Marlow et al., 2014; Tessmar-Raible et al., 2007) and/or in the anterior pole ectoderm of the larvae of the brachiopod *Terebratalia transversa* (Santagata et al., 2012). A striking feature of the anterior medial head of the centipede is the *FoxQ2* domain which is entirely nested within the *six3* domain (Fig. 1K), an arrangement which is found in the anterior pole ectoderm of free swimming larvae of diverse marine organisms such as brachiopods (lophotrochozoa), sea urchins and cnidarians (Santagata et al., 2012; Sinigaglia et al., 2013; Yaguchi et al., 2008), but has so far not been observed in any arthropod.

Based on these gene expression patterns and the lack of segmental gene expression within the anterior medial head we propose that this developmental territory is indeed non-segmental and homologous to the apical pole ectoderm of lophotrochozoan larvae. The presence of a similar territory in deuterostome larvae and in the radially symmetric cnidarian larva indicates that this anterior developmental territory is inherited from the bilaterian ancestor (Sinigaglia et al., 2013).

A population of neural cells originates in the anterior medial head and pioneers the axonal tracts of the central nervous system

Based on the deep evolutionary origin of the non-segmental anterior-medial tissue, one would expect ancient parts of the brain to derive from this region. Therefore we tested whether the anterior medial region gives rise to neuronal cells that contribute to the brain and central nervous system.

The foundation of axonal pathways from an anterior medial cell population

Neurogenesis in *Strigamia maritima* has been characterized by Chipman and Stollewerk (2006). Neural progenitor cell groups, expressing the pro-neural gene *achaete-scute homologue (ash)* (Linne et al., 2012; Fig. 3D), invaginate in small clusters of 5–9 cells from the segmental ventral neuroectoderm. Once internalized these clusters differentiate into neural cells. In the trunk segments these pro-neural clusters show a regular bilaterally symmetric arrangement in 7 rows of 3–6 invagination sites per hemisegment. Invagination sites with similar characteristics are also present in the head segments, including the pre-antennal region, but are not arranged in an obviously stereotypical pattern (Chipman and Stollewerk, 2006).

In the anterior medial region of the head no such invagination sites are visible, and *ash* is not expressed in this medial region during early development (see Fig. 3D). However, Chipman and Stollewerk (2006) noted that, prior to the invagination of the pro-neural cell groups, early axonal tracts were already present

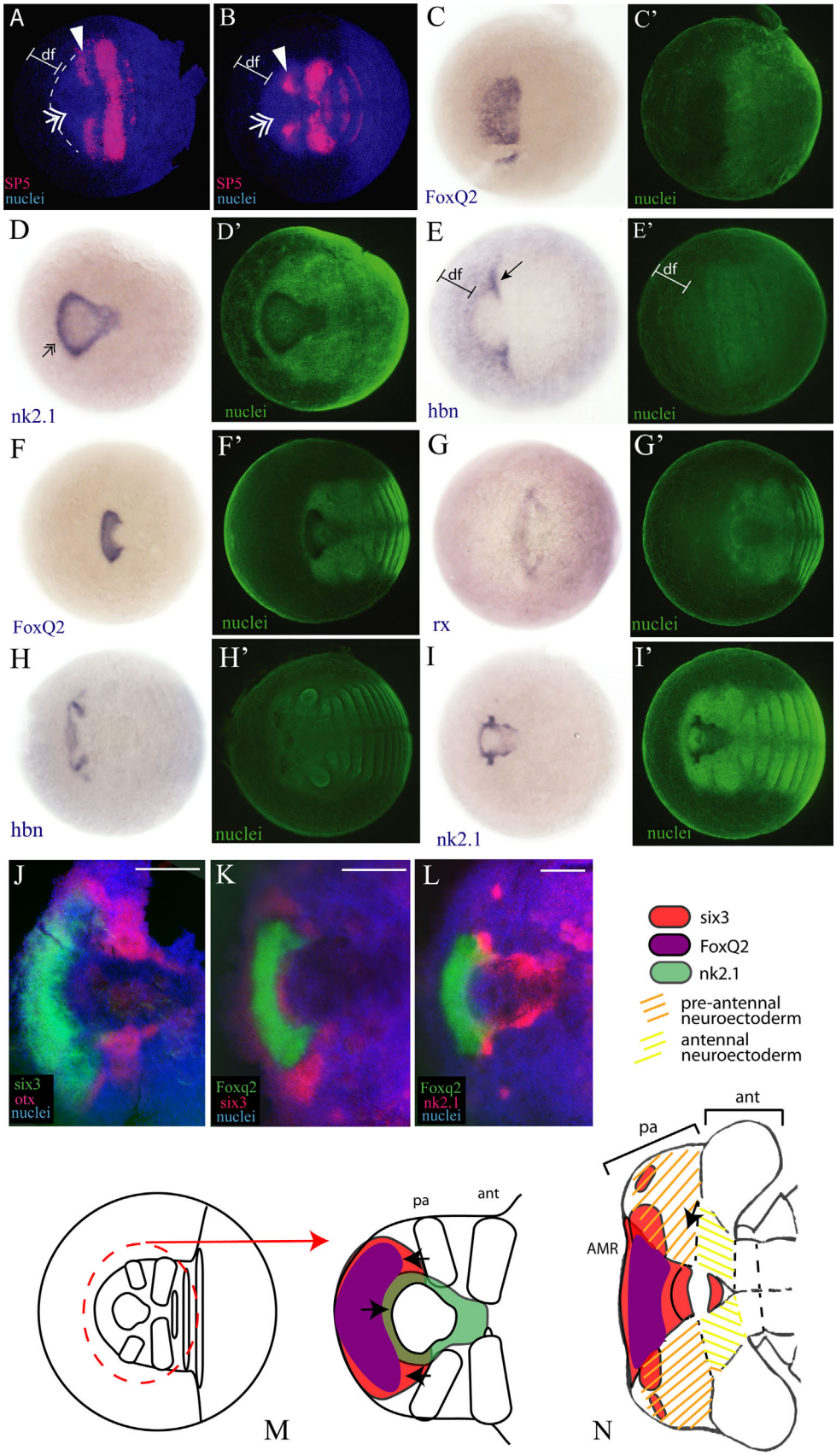

Fig. 1. Morphogenesis of the head field and early gene expression within the anterior medial tissue. A-L *In situ* hybridisation, probes as indicated in the pictures. **A** (head condensation, stage 2.3), **B** (late head condensation/early segmentation, stage 3.1): Expression of *SP5* marks the segmental territories of the early head. White arrow points at expression in the pre-antennal region. Double headed arrow points to the anterior medial tissue. Dashed line in A marks the anterior margin of the condensed head field. **C-E:** Expression of *FoxQ2*, *nk2.1* and *hbn* during early head condensation. **F-I:** Expression domains of anterior medial markers (now including *rx*) during early segmentation stage (Stage 4.1–4.2). C', D', E', F', G', H', I' show nuclear stain of the specimens. **J:** *six3* expression in AMR is directly anterior to pre-antennal *otx* expression. **K:** Nested domains of *FoxQ2* and *six3* in AMR. **L:** *FoxQ2* overlaps with the anterior portion of *nk2.1* expression (compare to I). **M, N:** Schematic drawings of the anterior medial domain, and its inclusion between the two halves of the pre-antennal region. **M -** head condensation stage; **N -**segmented germ band; **N** only includes *six3* and *FoxQ2* expression. pa=pre-antennal region, ant=antennal segment, AMR=anterior medial region. Bracket in A, B, E and E' marks the 'dorsal field' (**df**), the thin epithelium covering the anterior hemisphere which may be extra-embryonic. A-I=whole embryo diameter is between 1.1-1.2 mm; scale bars J-L=100 μm.

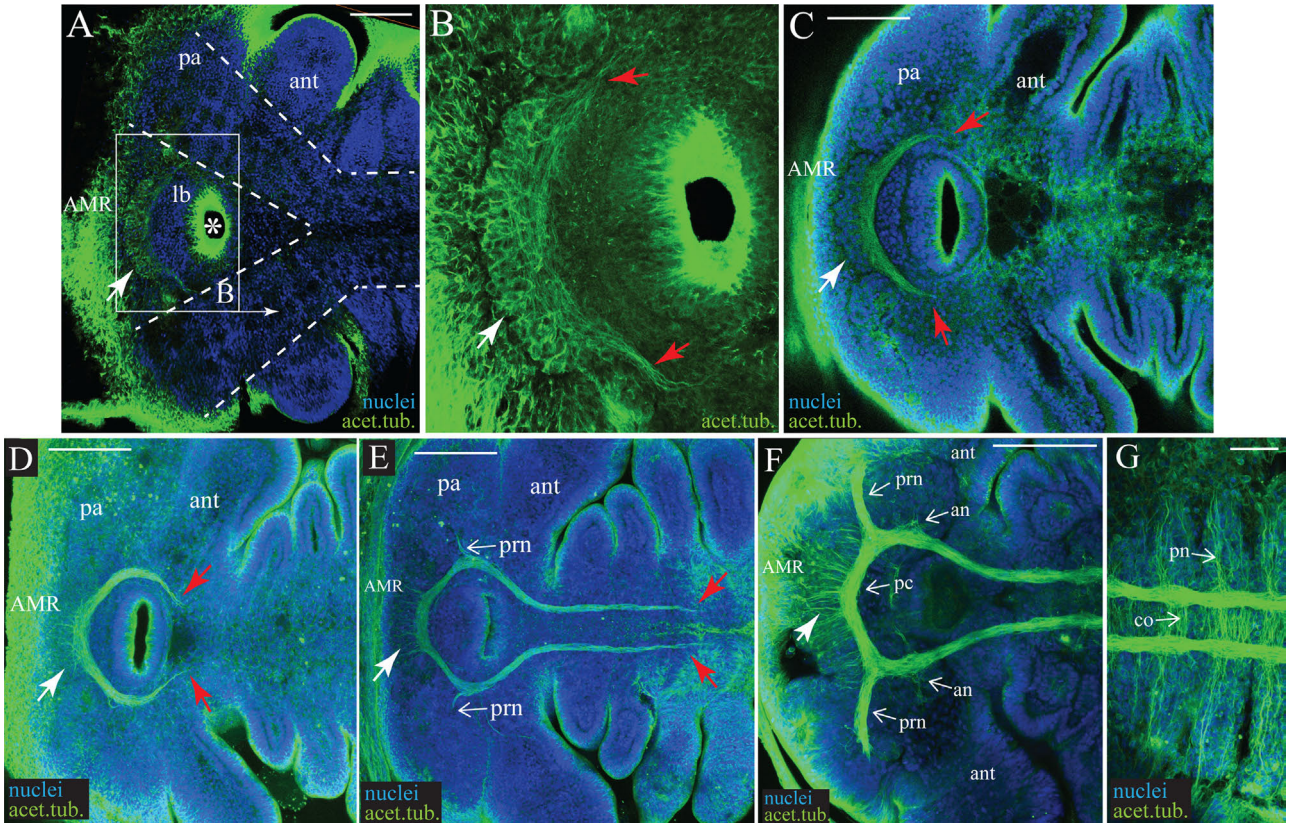

Fig. 2. Axons originating from an anterior medial cell population erect the primary scaffold of the central nervous system. Labelling of the nervous system with an anti-acetylated tubulin antibody, developmental series. Reconstructions of confocal microscope image stacks**. A:** Early segmentation (stage 4) embryo. White arrow points at neurogenic cell population in AMR; the ventral neuroectoderm with invaginating cell clusters lies in the Y-shaped area between the dashed lines. A white asterisk marks the stomodaeum **B:** White arrow points at cell bodies, red arrows at elongating ends of the axonal bundles **C-E:** Elongation of axonal bundles (red arrows) during stages 4.3-5; cell bodies remain in anterior medial position. **F:** Elaboration of the anterior nervous system, brain development. **G** Addition of commissures and peripheral neurons to the longitudinal axonal tracts. AMR=anterior medial region, lb=labrum, pa=pre-antennal segment, ant=antennal segment, **prn**=pre-antennal neuropil, **pc**=protocerebral commissure, **an**=antennal nerve, **pn**=peripheral neurons, **co**=commissure. Scale bars: A, C, D-F=100 μm, G= 50 μm.

beneath the surface ectoderm of trunk segments. This suggested the presence of pioneer axons that build up an early scaffold for the following development of the nervous system. Retrograde labelling experiments by Whitington et al. (1991) showed in a scolopendromorph centipede, *Ethmostigmus rubripes*, that cell bodies of longitudinal axons of the developing central nervous system are located in the brain, anterior to the stomodaeum.

We labelled the developing nervous system of *Strigamia* with an antibody against acetylated tubulin and found that cells located in the anterior medial head are the first cells to differentiate into neurons (Fig. 2A and B). These cells form a dense cluster and project axons in posterior direction (Fig. 2A–C). Directly posterior-basal of the cell bodies the axons fasciculate and at least some cross-over, so that cells from the right side send their axons to the left body half and *vice versa.* This is evident from the undivided architecture of the anterior-most brain commissure, which can only be achieved by midline crossing of some axons. (Fig. 2B). The neurons then elongate and form the early longitudinal axonal pathways along the AP axis of the embryo (Fig. 2C and F). Later in development these tracts become more prominent (Fig. 2F and G), presumably through a secondary contribution of processes from the pro-neural cell clusters of the segmental neuroectoderm. Subsequently peripheral neurons and transverse commissures that connect the left and right longitudinal strands differentiate from the segmental neuroectoderm (Fig. 2G).

The first neurons to deviate from the primary axonal tracts project bilaterally into the pre-antennal region (Fig. 2 E–F). We call these projections 'lateral protocerebral connections'; they later connect to lobes located in the pre-antennal region that, based on marker gene expression, most likely form the mushroom bodies (see Supplementary Fig. S4D–I). The part of the axonal scaffold lying directly basal to the anterior founder cell population is the protocerebral commissure, which connects the two longitudinal projections and crosses the anterior medial region (see Fig. 2F). At late developmental stages single cells are still connected via axonal projections to this anterior bridge and (Fig. 2F).

The anterior pioneer axons derive from the anterior medial region and are characterized by expression of the neural differentiation marker collier

We followed back the origin of the pioneer neurons by marker gene expression and found that they originate from the anterior medial part of the head. At stage 3, (early segmentation; (Brena and Akam, 2012)) a number of cells arranged in a bow within the surface layer of the anterior medial head region start to express *collier* (*col*) (Fig. 3A, B). In other animals *col* marks cells that are postmitotic and committed to a neural fate (Baumgardt et al., 2007; Demilly et al., 2011; Garcia-Dominguez et al., 2003). With on-going condensation of the head field these *col* positive cells form a dense cluster and sink under the surface epithelium (Fig. 3C). At the stage of specification (first expression of *col*) and internalization of these cells the factors *six3*, *FoxQ2*, *nk2.1*, *hbn* and *rx* are expressed in the territory from which the cells derive (see Fig. 1 and diagram in Fig. 7A,B). Double labelling against *col* gene expression and acetylated tubulin shows that the *col* expressing cells are indeed identical with the axonal pioneers (Fig. 3D).

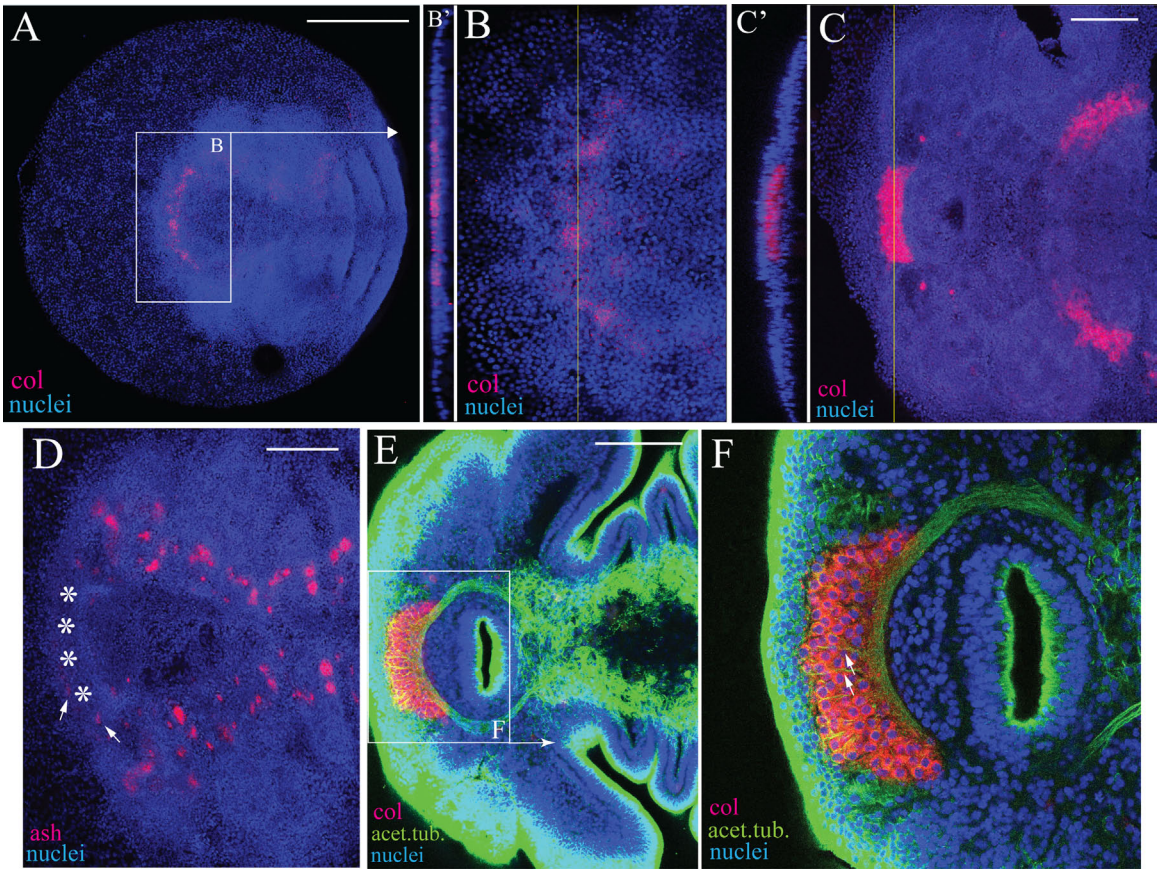

Fig. 3. The neural cells originating in the AMR express *collier*. A-D *In situ* stains of gene expression, developing nervous system. Reconstructions of confocal microscope image stacks**. A-B** (early segmentation, stage 3.2)**:** *Col*+ cells appear in a loosely arranged arch in the AMR. *Col*+ cells are in the surface cell layer (see B'). **C** (stage 4.1): *Col*+ cells have sunk beneath the surface (see C') and form a dense cell cluster. Yellow lines in B and C indicate planes of orthogonal section in B' and C'. **D** (stage 3.2): The pro-neural gene *ash* is expressed in cells of the lateral neuroectoderm. The central AMR where the *col*+ cells are situated (marked by white asterisks) is mostly free of *ash* expression. At this stage expression is only seen in few cells at the border of the AMR (marked by white arrows). E, F (stage 4.3): *Col* expression in the cell bodies of the pioneering axons. White arrows in F point at axonal connections to the central neuropil. Scale bars: F=300 μm; C–F=100 μm.

The early differentiation and behaviour of these anterior medial neural cells differs from that of the pro-neural cell groups in the segmental neuroectoderm. They are at first arranged in a loosely organized but coherent group in the surface layer (Fig. 3B), not tight focal clusters. Unlike the segmental neuroectoderm, they do not express *ash* prior to their differentiation. Conversely, the segmental neuronal precursors do not express *col* before or during their internalization (Fig. 3D). These differences in development suggest that the neuronal cells of the AMR are not serially homologous to the neurons and segmental ganglia that develop from the posteriorly following segments. Another characteristic feature of the differentiation process in the anterior medial region is that, in contrast to the formation of brain parts arising from the cephalic segments and to the formation of segmental ganglia of the ventral nerve cord, it has no bilateral character; from the time of their first differentiation the cells are arranged in an undivided medial group.

The late expression domains of six3, irq-A, FoxQ2, hbn and rx substructure the anterior medial head around the group of pioneer neurons

By Stage 4.3 (Brena and Akam, 2012), when the anterior medial neuronal cells are specified and lie at the anterior tip of the axonal scaffold, the AMR is substructured by a specific expression profile of the regional patterning genes. *Six3* continues to be expressed throughout the whole anterior medial head, but its expression is significantly reduced in the group of medial neuronal cells. Cells lying ventral and dorsal to this cell cluster still show strong expression of *six3* (Fig. 4A, B). *FoxQ2* expression is nested within the *six3* expression domain, and by contrast to *six3*, it is expressed strongly within the medial neuronal cells (Fig. 4 D, E). In addition *FoxQ2* is expressed in two lobes lying lateral to this central cell population (which will give rise to the *pars intercerebralis,* see below) and its expression reaches posteriorly into the anterior lip of the stomodaeum (Fig. 4 D, E). A marker that is not expressed during early development of the AMR but is found in this tissue at this later stage (4.3) is *iroquois-A* (*irq-A*). The *Strigamia* genome contains three *iroquois* genes of which two, *irqB* and *irqC* are expressed in the anterior medial region but also within the entire segmental neuroectoderm (data not shown). *Irq-A* (most closely related to *Drosophila caupolican* and *araucan*) is expressed within the AMR but only dorsally in the cells overlying the medial neuronal cell cluster (Fig. 4F, G). Both *rx* and *hbn* are expressed only ventrally of the neurogenic cells, mutually exclusive with *irq-A* (Fig. 4G J). Neither *rx/hbn* nor *irq-A* is expressed in the medial neurogenic cells themselves. With proceeding development *rx* and *hbn* retreat from the anterior-most part of the AMR.

Development of the anterior neurosecretory protocerebrum

Neurosecretory activity of the neuronal founder cells

In the polychaete *Platynereis* the anterior *nk2.1/rx* positive region (which is also *six3* positive (Steinmetz et al., 2010)), gives rise to a neurosecretory fibre plexus that is located at the anterior tip of the axonal scaffold. Hence we suspected that the neurogenic

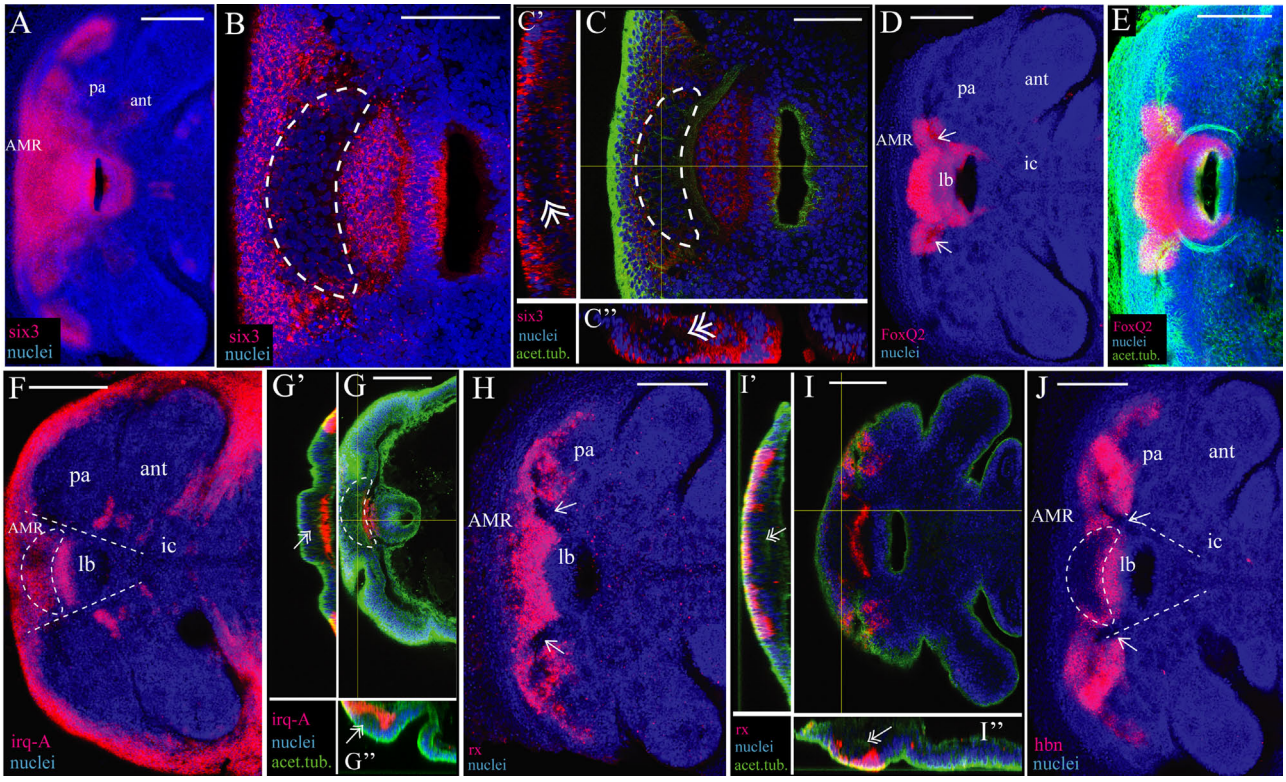

Fig. 4. Regional patterning of the AMR. *In situ* stains of gene expression, developing nervous system. Reconstructions of confocal microscope image stacks. All stage 4.3 embryos. **A, B, C:** Regional expression of *six3* in the entire AMR tissue, down-regulation of expression within the pioneer neuronal cell population (encircled in B, C). **D, E:** *FoxQ2* expression in a compact domain surrounding and including the anterior medial cells and the *pars intercerebralis* compartments (white arrows). F, G: Expression of *irq-A* in the anterior medial tissue is only dorsally (internally) in the embryo above the medial neuronal cells (see G', G″). H, I: Expression of *rx* in the AMR, ventral to the pioneer neuronal cells (see I', I″). J: *Hbn* is expressed in a pattern similar to that of *rx*; both are absent from the placodal invagination sites of the *pars intercerebralis* (white arrows); expression retracts from the AMR as development proceeds. Encircled area in B, C, F, G and J marks the position of the medial neural cell cluster. **AMR**=anterior medial region, **pa**=pre-antennal segment, **ant**=antennal segment, **ic**=intercalary segment **lb**=labrum. Scale bars: A, D–J=100 μm; B, C=50 μm.

cells that derive from a territory with similar molecular characteristics might form a neuroendocrine nucleus. We therefore tested whether they express *pro-hormone convertase 2* (*phc2*), an enzyme involved in neurohormone processing, which in *Platynereis* marks the anterior neurosecretory fibre plexus (Tessmar-Raible, 2007; Tessmar-Raible et al., 2007). The centipede *phc2* gene is expressed in exactly the same cells that express *col* (compare Figs. 3 E,F; 5 A, B) and pioneer the early axonal scaffold (see Fig. 2). Hence the pioneering neuronal cell population that derives from the centipede AMR is an early active neurosecretory centre that shares molecular and positional similarities with the neurosecretory fibre plexus in the polychaete (Tessmar-Raible et al., 2007).

Development of the centipede pars intercerebralis from bilateral invaginating head placodes

We wondered whether the neurosecretory cells that are specified in the anterior medial head give rise to the centipede *pars intercerebralis*, which in insects is the anterior neurosecretory centre of the central complex (De Velasco et al., 2007) and develops from the *six3* positive territory in *Drosophila* and in the beetle *Tribolium* (Posnien et al., 2011; Steinmetz et al., 2010). Characteristic of the developing insect *pars intercerebralis* is that it develops from head ectodermal placodes that invaginate from the surface and form ectodermal compartments inside the embryo (De Velasco et al., 2007).

Two bilateral pairs of placodal invagination sites are found in the anterior head of the centipede. The central ones, which we term the main head placodes, develop at the border between the anterior medial region and the pre-antennal region (Fig. 5E). A pair of smaller invagination sites of unknown fate forms more laterally in the anterior head (the lateral head placodes). The ectoderm of both pairs expresses *six3* (see Fig. 5F).

In *Drosophila* cells that form the *pars intercerebralis* express *Dchx*, the orthologue of vertebrate *vsx*, throughout development (De Velasco et al., 2007). We tested expression of the centipede *vsx* gene and found that it is expressed in cells that lie immediately lateral to the medial neurosecretory cells and marks the medial part of the ectodermal compartments that derive from the main head placodes (Fig. 5C, D, G). (The more lateral part of these placodes express mushroom body markers, see Supplementary Fig. S4E, F, I). Based on *vsx* expression and the mode of development from invaginating ectoderm we conclude that the medial parts of the main head placodes give rise to the centipede *pars intercerebralis*. The *vsx* expression domains are within the nested *six3* and *FoxQ2* expression domains (see Fig. 5A, D), but notably *hbn/rx* and *nk2.1* expression is absent from the invagination sites and ectodermal compartment of the *pars intercerebralis* (Figs. 1G, H, L, 4H J). The *pars intercerebralis* compartments are also devoid of *col* and *phc2* expression that mark only the central neurosecretory cells. Hence there are two distinct anterior structures that develop from the *six3/FoxQ2* domain, the unpaired median population of neurosecretory pioneer neurons and the bilateral *pars intercerebralis*.

Hypothalamus-like cell types in the central pioneer-neuronal/neurosecretory cell cluster and in the pars intercerebralis

The apical plate derived neurosecretory region of the polychaete shares molecular similarities with the neuroendocrine brain centre of vertebrates, the hypothalamus. Cells that give rise

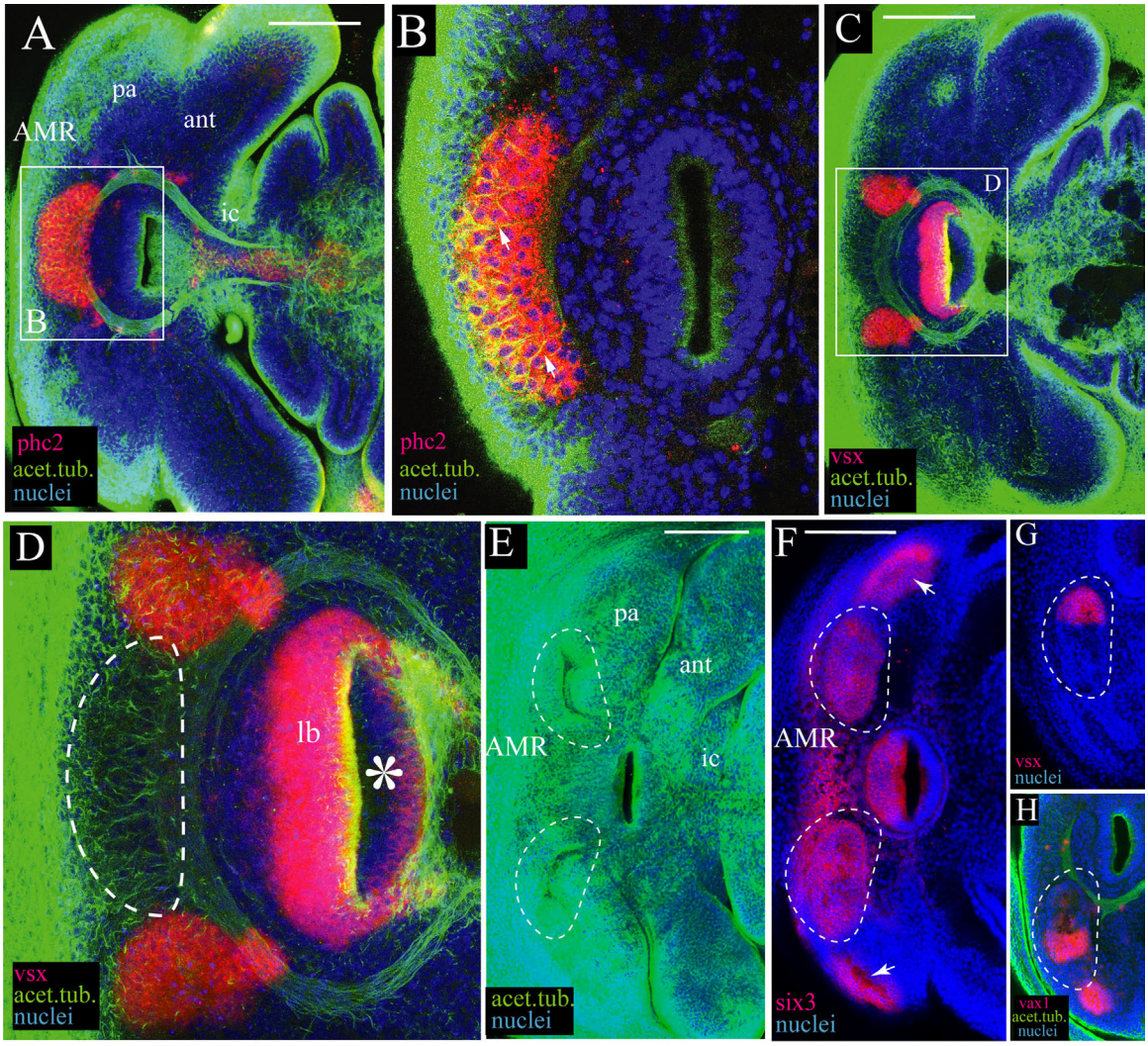

Fig. 5. Neurosecretory activity of the axonal pioneers and development of the *pars intercerebralis*. *In situ* stains of gene expression, developing nervous system. Reconstructions of confocal microscope image stacks. All stage 4.3 embryos. A, B: Expression of *phc2* within the medial neurogenic cells. C, D: Expression of the pars-intercerebralis marker *vsx* in developing brain structures situated directly lateral to the medial neural cells, and around the stomodaeum (white asterisk in D). E: Surface rendering using the stain of cytoskeletal acetylated-tubulin. Invagination sites of the main head placodes are visible (encircled areas). The medial part of the invagination site co-localizes with *vsx* stain shown in C and D. F: *Six3* is expressed in the sub-surface ectoderm that derives from the main head placodes (encircled areas) and the lateral head placodes (white arrows). **G** *Vsx* is expressed only in the medial part of the main head placode-derived ectoderm (encircled). *Vax1* is expressed only in the lateral part of the main head placode-derived ectoderm (encircled), mutually exclusive with *vsx*. **AMR**=anterior medial region, **pa**=pre-antennal segment, **ant**=antennal segment, **ic**=intercalary segment, **lb**=labrum. Scale bars A, C, E, F=100 μm.

to the zebrafish hypothalamus derive from a *six3*, *nk2.1* and *rx* positive region at the anterior end of the neural plate, which is reminiscent of the neurosecretory plexus formation in the polychaete (Steinmetz et al., 2010; Tessmar-Raible et al., 2007). Based on this Tessmar-Raible and others have proposed that the anterior neurosecretory cells in both these territories likely share a common evolutionary origin (Marlow et al., 2014; Steinmetz et al., 2010; Tessmar-Raible, 2007; Tessmar-Raible et al., 2007).

To test for similarities in the molecular identity of vertebrate hypothalamus neurons and the anterior neurosecretory cells of *Strigamia* we examined some of the factors that are known to mark hypothalamic neurons. The gene *ventral anterior homeobox 1* (*vax1*) is involved in formation of the pituitary gland and also defines rostral hypothalamic progenitors in the forebrain (Bertuzzi and Hindges, 1999; Bharti et al., 2011; Wataya et al., 2008). *Vax1* genes are absent from sequenced insect genomes and so far no *vax1* gene has been characterized in any arthropod. We found a clear *vax1* orthologue in the centipede genome. Embryonic expression of *vax1* in the centipede is restricted to the anterior-most part of the head (Fig. 6A, B). In the medial domain the pattern is punctate and marks single cells, which are located within the medial neurosecretory cell cluster (Fig. 6B). The lateral expression of *vax1* is within the compartmentalized ectoderm that derives from the head placodes. It is however absent from the medial part of the main head placode that expresses *vsx* and produces the *pars intercerebralis* (Fig. 5H).

A second factor that is required for the specification of neurosecretory hypothalamic neurons (Wang and Lufkin, 2000) is *orthopedia* (*otp*). We find expression of the centipede *otp* gene in a punctate pattern concentrated within the anterior medial region. *Otp* positive cells are found in both the medial neurosecretory cell population and the *pars intercerebralis,* and also more laterally (Fig. 6C, D).

We identified within the centipede genome a single *vasotocin (vasopressin/oxytocin) -neurophysin* (*vtn*) orthologue. The vertebrate orthologues encode neuropeptides secreted by the periventricular hypothalamus (Pearson and Placzek, 2013). This gene has been lost from the *Drosophila* genome but is present in other insects, including at least some orthopterans and the beetle *Tribolium* (named *inotocin* in insects; Stafflinger et al., 2008). The

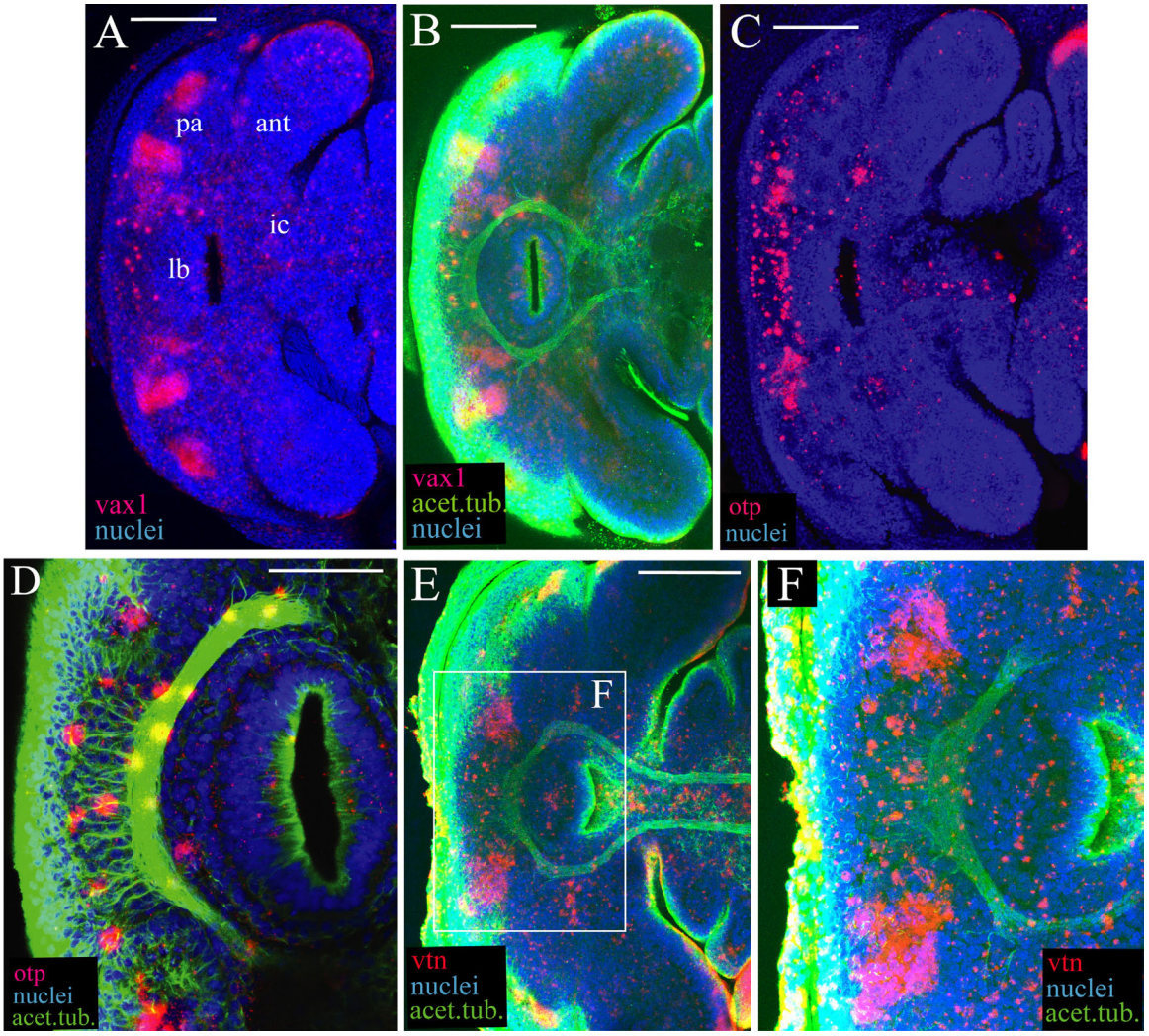

Fig. 6. Expression of the hypothalamic marker genes *vax1*, *otp* and *vtn* in the anterior medial region. *In situ* stains of gene expression, developing nervous system. Reconstructions of confocal microscope image stacks. A-D stage 4.3, E-F stage 5 embryos. A, B: Expression of *vax1* in some of the medial neural cells and laterally in the placodal derived ectoderm, but not in the *pars intercerebralis* (compare 5D). C: Expression of *otp* in a punctate pattern within the anterior head. D: *Otp* is expressed in some of the cells belonging to the medial neural cell cluster. E, F: Expression of the RNA encoding the neuropeptide *vtn* is detected at higher levels within cells of the medial neural cell cluster and *pars intercerebralis* than in the remaining ectoderm. **oc**=ocular segment, **pa**=pre-antennal segment, **ant**=antennal segment, **ic**=intercalary segment, **lb**=labrum. Scale bars: A-C, F=100 μm; D=50 μm.

expression data for *vtn* in the centipede is not as clear as for transcription factors (perhaps because the levels of expression are low at these embryonic stages), and long staining times led to increased background. Nevertheless we detected transcripts of the centipede *vtn* gene in a punctate pattern throughout the ventral neuroectoderm and ventral midline, with more concentrated expression within the anterior medial neurosecretory cell population, and also within cells of the *pars intercerebralis* (Fig. 6E, F). This is similar to the *otp* expression pattern, which is consistent with findings in other animals that both factors are co-expressed and that *otp* is involved in the regulation of vasopressin/oxytocin expression in the mouse hypothalamus (Acampora et al., 1999; Tessmar-Raible, 2007).

Thus the medial neurosecretory cells of the centipede share similarities with both the polychaete neurosecretory plexus and the vertebrate hypothalamus. These medial cells occupy an anterior position within the nervous system and originate from an *nk2.1+/rx+/six3+* territory. They contain cells that express the transcription factors *vax1* and *otp*, which specify hypothalamic neurons (Wang and Lufkin, 2000; Wataya et al., 2008) and cells that express the hypothalamic neuropeptide *vtn*. The *pars intercerebralis* is developmentally distinct from the medial cells and does not have a pioneering function in nervous system development. It also derives from a territory that expresses *six3* (and *FoxQ2*), but is devoid of *vax1, nk2.1* and *rx*. The latter two factors are characteristic of the hypothalamus progenitor tissue (Pearson and Placzek, 2013; Tessmar-Raible et al., 2007). However, cells expressing markers of hypothalamic neurons, *vtn* and *otp*, are also found in the developing *pars intercerebralis*.

Discussion

We have identified an anterior cell population in centipedes that lies medial to the primordia of the *pars intercerebralis*, and which appears to be without a recognised counterpart in insects (but see below). The fate of these cells in the adult brain is unclear, but given that they establish the anterior commissure, they are likely to contribute to the centipede central complex.

The central complex is well defined in insects, where it consists of different elements including the central body, ellipsoid body and a protocerebral bridge (Boyan and Reichert, 2011; Strausfeld, 2012). In millipedes the midline neuropil of the central complex is greatly reduced, but a distinct midline neuropil corresponding to

the insect central body has been found in several centipede species (Loesel et al., 2002; Strausfeld, 2012). It is situated between the mushroom bodies and is innervated by allatostatin-like immunoreactive peptidergic cells (Loesel et al., 2002). The anterior neurosecretory pioneer cells that we identify in Strigamia show several similarities to this central body. They lie between the protocerebral lobes anterior to the first developing brain commissure and the initial fibres originating from their cell bodies project longitudinally in a parallel array, before joining the anterior commissure.

In insects it has been shown that most elements of the central complex, including the central body, are produced by neuroblasts located within the *pars intercerebralis* (see Boyan and Reichert, 2011). Knockdown of *six3* function in the beetle *Tribolium* disrupts central body formation (Posnien et al., 2011), supporting the idea that the central body derives from the *six3* positive territory. In the centipede both embryonic structures, *pars intercerebralis* and medial cell cluster, are in close proximity within the *six3* (and *FoxQ2*) expressing territory. Therefore it seems likely that both cell populations produce the central body neuropil of *Strigamia*.

Anterior midline neuropils are found in most arthropods and in onychophorans (Strausfeld, 2012), but little is known about their embryonic development outside the insects. In the spider *Cupiennus salei* the arcuate body, which is a possible homologue to the insect central body (Loesel et al., 2011; Strausfeld, 2012), is formed by bilateral invaginations of the protocerebral ectoderm and a subsequent fusion at the midline. In addition many postmitotic neural precursor cells located in the medial pre-cheliceral domain contribute to to the central protocerebrum (Doeffinger et al., 2010). The lateral invaginations bear similarities to the invaginating 'head-placodes' of the centipede. It is however not clear whether the medial neural precursors are similar to the anterior medial cells of *Strigamia*. More comparative developmental work is required to elucidate the evolutionary relationships of embryonic cell populations across the arthropods, and of the adult structures that they give rise to.

The anterior pole of the head axis and the anterior-posterior organisation of the protocerebrum

The interpretation of the anterior neuro-axis in arthropods is still subject to dispute. Some authors have interpreted structures that derive from the pre-antennal (ocular) region, in particular the eyes, as the anterior-most tip of the neural axis of arthropods (Haas et al., 2001; Rempel, 1975; Siewing, 1963), but molecular work in insects hints at the central complex as being the anterior-most brain structure (Posnien et al., 2011; Urbach and Technau, 2003). In 1963 Siewing proposed a subdivision of the protocerebrum into archicerebrum comprising the ocular lobes and the mushroom bodies, and prosocerebrum, comprising the central complex. He interpreted the archicerebrum as the anterior-most part of the brain (Siewing, 1963; discussed in Scholtz and Edgecombe, (2006)). Urbach and Technau (2003) also suggested a subdivision of the protocerebrum into archi- and prosocerebrum, but see the *pars intercerebralis* and the central complex, which is at least partially formed by progenitors located in the developing *pars intercerebralis* (Boyan and Williams, 2011; De Velasco et al., 2007; Williams and Boyan, 2008) as the anterior-most brain structures. This is based on a map of neuroblasts in the head, where cells that give rise to the *pars intercerebralis* are located in the anterior-most, medial part of the insect head lobes (Urbach and Technau, 2003). Similarly Strausfeld 2012 argues that the *pars intercerebralis* is part of an ancestral, rostral and a-segmental brain (Strausfeld, 2012).

Our work in the centipede now clearly supports an embryonic origin of the protocererebrum from two developmentally distinct regions, an ocular/preantennal region, and the anterior medial region. These regions are characterised by the expression of largely non-overlapping sets of transcription factors. Brain structures that derive from the AMR include the *pars intercerebralis*, well documented in insects and other arthropods, and the anterior *col*+ medial/neurosecretory cell cluster, which has not previously been described in insects or any other arthropod. Together these structures represent the anterior tip of the neural axis, as Urbach and Technau, and Strausfeld, proposed. Brain parts deriving from the ocular/pre-antennal region are more posterior structures.

The evolutionary origin of the anterior-most part of the centipede protocerebrum and origin of the axonal scaffold from an apical-organ like neurosecretory cell population

The anterior-medial neurosecretory cell cluster derives from a region expressing a broadly conserved suite of transcription factors. Central to this system is a domain of *FoxQ2* expression nested within *six3* expression. In many marine larvae, the cells of the apical organ are specified centrally within this *FoxQ2* domain, a pattern that has been found in organisms as diverse as brachiopods, polychaete annelids, cnidarians, echinoderms and hemichordates (Darras et al., 2011; Lowe et al., 2003; Marlow et al., 2014; Santagata et al., 2012; Sinigaglia et al., 2013; Wei et al., 2009; Yaguchi et al., 2008; Yankura et al., 2010). This molecular topography has been particularly well characterised in the anterior pole ectoderm of the Brachiopod *Terebratalia transversa* (Santagata et al., 2012). This reveals parallels with *Strigamia* also in dorso/ventral organisation: Expression of *hbn* in *Terebratalia* is restricted to the ventral side of the animal, as in *Strigamia*.

Despite the apparently ancient origin and conserved molecular fingerprint of this anterior territory (the apical plate), we did not anticipate finding a cell population homologous to the apical-organ itself in the centipede, because neither primary free-swimming larvae nor ciliated epithelia are present in ecdysozoans, and nothing recognisable as an apical organ has been reported in any extant arthropod (Telford et al., 2008). However, although the larval apical organ degenerates completely during metamorphosis in those groups such as sea urchins and cnidarians, that undergo a complete reorganisation of the body plan at metamorphosis (Nielsen, 2005, and literature cited therein), there are other groups in which the apical organ, and other structures derived from the apical plate, integrate into the axonal scaffold of later larval and adult stages (Fischer et al., 2010; Santagata et al., 2012). This would be typical, for example, of annelids, which show a continuity of function from trochophore to larva to adult. It is now clear that in at least some Spiralia, cells of the apical organ form a neuropil that contributes to the larval and sometimes even to the adult central nervous system (Fischer et al., 2010; Santagata et al., 2012; Tessmar-Raible et al., 2007) and execute important neuroendocrine functions (Conzelmann et al., 2013, 2011; Tessmar-Raible et al., 2007). This makes the finding of a similar cell population persisting within some arthropods less surprising.

Whether or not ecdysozoans evolved from an ancestor with a free-swimming larval stage, our results suggest that the common ancestor of ecdysozoans and lophotrochozoans possessed an anterior domain characterised by a conserved regulatory signature that gave this anterior tissue the competence to form neural/neurosecretory cells. Either arthropods lost the ability to form a ciliated apical tuft from cells within this territory (as they lost ciliation in general), or the ciliated tuft might have been acquired independently during the evolution of larval forms.

Anterior pioneer neurons in centipedes, insects and crustaceans

Our results suggest that the centipede anterior medial cell population, which expresses the apical organ markers *col*, *phc2*

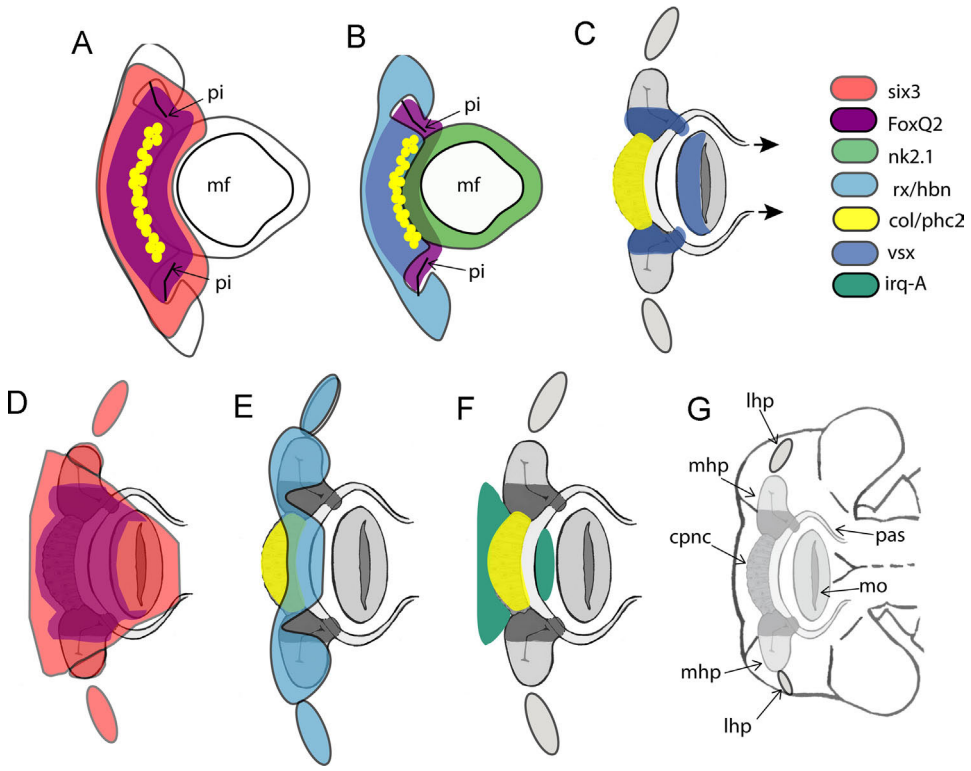

Fig. 7. Summary of structure and gene expression characterizing subdomains of the AMR tissue. Schematic drawings. A and B: Early gene expression domains (at late head condensation/early segmentation stage) anterior to the mouth field (mf). *Col* expressing neural cells are specified within a domain of nested *six3* and *FoxQ2* expression and adjacent *rx/nk2.1* expression. Black lines indicate invagination sites of the *pars intercerebralis* (pi) C–F: Gene expression domains in relation to the developing nervous system. *Col+/phc+* cells are located at the anterior end of the primary axonal scaffold, laterally bordered by the *vsx*-expressing *pars intercerebralis* and surrounded by *six3*, *FoxQ2* and *irq-A* expression. Black arrows in C indicate the extension of the primary axonal scaffold in posterior direction, **G:** Architecture of the developing protocerebrum and anterior axonal scaffold. **pi**=pars intercerebralis placodal invagination site, **mf**=mouth field, **lhp**=lateral head placode, **mhp**=main head placode, **cpnc**=central pioneer neuronal cells, **mo**=mouth, **pas**=primary axonal scaffold.

and *otp* (Conzelmann et al., 2013; Jackson et al., 2010; Marlow et al., 2014; Pang et al., 2004; Santagata et al., 2012; Tessmar-Raible et al., 2007) serves an important function in erecting the primary scaffold of at least the anterior central nervous system. Similar long range pioneer neurons originating from the anterior pole have not so far been characterized in any other arthropod. In most insects for instance the neuroblasts that pioneer the axon tracts of the anterior nervous system are located in the bilateral head neuroectoderm (Posnien et al., 2011; Urbach and Technau, 2003; Younossi-Hartenstein et al., 1996).

Interestingly two short range pioneer neurons differentiate within the anterior medial domain of the head of the grasshopper *Schistocerca gregaria* (Boyan and Williams, 2008; Ludwig et al., 1999), an insect that forms most of its nervous system through stem-cell like progenitor cells (Shepherd and Bate, 1990). These two cells originate directly from the epithelium and not from intermediate progenitors. They are the pioneers of the primary brain commissure of the grasshopper (Boyan and Williams, 2008; Ludwig et al., 1999). Although this alternative mode of formation of the brain commissure is restricted to single pioneer cells, it shows intriguing similarity to the development of the pioneer neurons that are directly specified within the surface epithelium of the *Strigamia* anterior medial head, and are quite distinct from the invaginating pro-neural cell clusters of the ventral neuroectoderm, which are the myriapod equivalent of the insect neuroblasts (Chipman and Stollewerk, 2006; Dove and Stollewerk, 2003; Stollewerk and Simpson, 2005). It would be exciting to see whether these cells arise from a territory in the grasshopper that expresses similar molecular markers as the *Strigamia* AMR.

Anterior medial cells with a neurogenic character have also been reported in some but not all crustaceans. In the amphipod *Orchestia cavimana* one of the first signs of axogenesis is that about six cells in a medial domain arrange in a row and contribute to the anterior protocerebral commissure (Ungerer et al., 2011). Pioneering neurons that have their origin in the brain have also been described in the crayfish *Cherax destructor* whereas in another malacostracan crustacean, the woodlouse *Porcellio scaber,* the first neurons have a segmental origin (Whitington et al., 1993). These authors do however comment that the pioneering axons from the brain in the crayfish are in their morphology not similar to the centipede pioneer axons (Whitington et al., 1993, 1991).

None the less, based on the conserved molecular characteristics of the anterior medial pioneer neurons in centipedes, we believe that this cell population probably does go back to the arthropod ancestor. It is possible that this ancestor possessed long range anterior axonal pioneers like the centipede, which during evolution have gradually been replaced by neurons from the segmental neuroectoderm. On the other hand it is also possible that ancestrally the axons from the medial domain only contributed to the anterior part of the axonal scaffold, as do the medial cells in the grasshopper (Boyan and Williams, 2008; Ludwig et al., 1999) and that they have been modified to long range pioneers in the myriapod lineage.

Conservation of anterior neurosecretory brain centres

There is some disagreement surrounding the structure of the vertebrate anterior neural plate, but the rostral hypothalamus probably marks its anterior-most tip (Puelles and Rubenstein, 2003; Rubenstein and Shimamura, 1998). Similarities in the markers expressed in the rostral hypothalamus and in the apical plate/apical organ of polychaete annelids have led Tessmar-Raible

et al. to propose that these structures share a common evolutionary origin (Tessmar-Raible et al., 2007). Our results argue that the centipede retains a derivative of the same ancestral structure.

Three of the regional transcription factors that characterize the AMR of the centipede, *six3*, *rx* and *nk2.1* are expressed in the medial forebrain region of vertebrates that produces the hypothalamus (Lagutin et al., 2003; Muranishi et al., 2012; Tessmar-Raible et al., 2007). In addition, several genes that mark neurosecretory cell populations in the hypothalamus are also expressed within the centipede anterior medial neurosecretory cells. For example, *otp* is required for the correct development of the hypothalamus from the rostral neural plate and for secretion of the neuropeptides arginine-vasotocin, oxytocin (both orthologous to *Strigamia vtn*), somatostatin and corticotropin releasing hormone (Wang and Lufkin, 2000). We found scattered cells in the medial population expressing *otp*, and a concentration of *vtn* expressing cells within the anterior medial population and the *pars intercerebralis* of *Strigamia. PC2* (*phc2*), which distinctively marks the anterior medial cells that pioneer the axonal scaffold of *Strigamia,* is expressed in the paraventricular and arcuate nuclei of the hypothalamus (Nillni, 2007). In addition one of the three mouse orthologues of *col*, *Olf-1/EBF-like 3,* is expressed in some cells of the hypothalamus (Wang et al., 1997). *Vax1*, which is expressed in a subset of the anterior medial pioneer neurons in the centipede, is required for axonal tract formation in the ventral forebrain and is prominently expressed in the presumptive hypothalamus (Bertuzzi and Hindges, 1999).

In conclusion, there is substantial overlap in the sets of markers that characterize the anterior medial neurosecretory cells of *Strigamia* (and other invertebrates) and the developing hypothalamus of vertebrates.

In mouse embryos *vax1* is also involved in formation of the pituitary gland. Its absence in the anterior-most ectoderm seems to be required for the invagination of pituitary gland progenitor tissue, as the complete lack of *vax1* in the anterior ectoderm leads to a second invagination further posterior (Bharti et al. 2011). This bears some similarity to the absence of *vax1* in the *pars intercerebralis* tissue, and its expression in surrounding areas.

In general, the development of the placodal invaginations in the centipede bears shows some parallels to anterior development in vertebrates where the medial forebrain domain is bordered by the invaginating adenohypophyseal and olfactory placodes (Schlosser et al. 2014). However, understanding a possible evolutionary relationship between all these structures would require further work focussing on the lateral invaginating head ectoderm of the centipede.

These results together suggest that the anterior neurosecretory brain centre of the bilaterian ancestor already possessed a relatively high degree of complexity and cell type diversification, a principle that also emerges from recent studies in cnidarian and lophotrochozoan larvae (Conzelmann et al., 2013, 2011; Marlow et al., 2014, 2009; Tessmar-Raible et al., 2007).

The development and structure of the anterior neurosecretory system seems to be less conserved in the insects used as major experimental models. The *pars intercerebralis* is conserved and developmentally well characterized in insects (De Velasco et al., 2007), but no cell population corresponding to the *collier*-expressing pioneer cells has so far been characterized in *Drosophila* or in *Tribolium*. In these insects, the cells of the *pars intercerebralis* merge to form the most medial neurosecretory structure (De Velasco et al., 2007), whereas in *Strigamia*, the bilateral parts of the *pars intercerebralis* remain separated by the *collier* expressing population. Both the medial pioneer cells and the *pars intercerebralis* derive from the *FoxQ2* and *six3* expression domain, and both express some of the hypothalamic cell type specific marker genes (*otp, vtn*). Hence both might originate from an ancient anterior neuroendocrine system that has diversified during evolution of the arthropod brain.

Experimental procedures

Embryo collection and fixation

Embryos were collected from a wild population near Brora, Scotland (Chipman et al., 2004a). The material was fixed for several days in 4% Formaldehyde/0.5 x PBS (details can be found in (Brena and Akam, 2012)). Embryos were stages according to morphological features as described in (Brena and Akam, 2012).

Gene identification and Cloning

Genomic resources for *Strigamia maritima* are available at http://www.ncbi.nlm.nih.gov/assembly/322118/. Gene orthologues were identified by *Blast* searches against the genomic and transcriptomic sequence. Gene identities were validated by reciprocal searches of the sequences against generic databases. Models of all identified head patterning genes were annotated on the genome and can be found at http://metazoa.ensembl.org/Strigamia_maritima/. Ensembl gene IDs are listed in the supplementary material (Supplementary Table S1).

In addition, for the characterization of *FoxQ2* and *irq-A* genes, phylogenetic trees (Supplementary Figs. S2 and S3) were created using Phylemon2 (Sánchez et al., 2011). Multiple sequence alignments were performed on protein sequences using MUSCLE (Edgar, 2004) and gene trees were built by maximum likelihood analysis in PhyML (Guindon and Gascuel, 2003). Tree calculation parameters are given in the accompanying material (Supplementary Figs. S2 and S3). A classification of the centipede neuropeptides and homeobox genes can also be found in the *Strigamia* genome publication (Chipman et al., in press).

Specific primers were designed against the identified gene sequences and products were amplified by standard PCR reaction and subsequently cloned into the pGEM-T-Easy vector system (Promega). Inserts were verified by sequencing and then used as templates for *in situ* probe synthesis.

In situ staining of gene expression and antibody stain of the central nervous system

Single and double colorimetric *in situs* were performed as described in (Chipman and Stollewerk, 2006; Chipman et al., 2004b). For *in situ* stains in conjunction with antibody stain against acetylated tubulin, embryos were pre-treated in a buffer containing 5% mercaptoethanol and 0.3% Triton (Yoshida-noro et al., 2000) to increase tissue permeability and allow increased penetration of the antibody. Permeabilised embryos were first taken through the probe incubation steps of the *in situ* hybridisation protocol and then incubated in the primary antibody (from mouse, clone 6–11-B1, Sigma) (1:250 v/v) at 4° over night. Embryos were washed several times in PBT (PBS+0.1% Tween-20) and then incubated with the secondary antibody (A488 goat anti mouse IgG, Molecular Probes) (1:500 v/v) at 4° over night. After several washes in PBT embryos were post-fixed for 10 min in 4% Formaldehyde. Finally embryos were incubated in the anti-DIG-AP antibody (1:3000 v/v) and a Fast Red (Roche) staining reaction was carried out. All embryos were counterstained with the nuclear dye Hoechst (H33342; used at 1/1000 v/v). A detailed protocol is available on request from the authors.

Image acquisition

Specimens were mounted in 90% Glycerol and analysed using a Leica SP5 upright confocal laser scanning microscope. The fluorescing properties of Fast Red (Murdoch et al., 1990) were used for laser scanning detection of the *in situ* stain using a 543nm He-Ne laser. The A488 labelled acetylated tubulin was visualized using an Ar laser at 488nm and for Hoechst detection we used a 405nm diode. For analysis and reconstruction of the image stacks we used the free software bundle FIJI (Schindelin et al., 2012). Brightness and contrast of images were adjusted using Photoshop CS5 (Adobe).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.09.020.

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